Review
Herbicide safeners: a review

Joanna Davies* and John C Caseley

IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol, BS41 9AF, UK

Abstract: Herbicide safeners selectively protect crop plants from herbicide damage without reducing activity in target weed species. This paper provides an outline of the discovery and uses of these compounds, before reviewing literature devoted to defining the biochemical and physiological mechanisms involved in safener activity. Emphasis is placed on the effects of safeners on herbicide metabolism and their interactions with enzyme systems, such as cytochrome P_{450} mono-oxygenases and glutathione-*S*-transferases. Attention is drawn to the potential wide-ranging applications of safeners and, in particular, their use as powerful research tools with which to identify and manipulate those mechanisms which contribute to herbicide selectivity and resistance.

Keywords: herbicide metabolism; herbicide selectivity; herbicide resistance; cytochrome P_{450}; glutathione-transferase

1 INTRODUCTION

Herbicide safeners selectively protect crop plants from herbicide damage without reducing activity in target weed species. These compounds have generated considerable commercial interest and research activity since their discovery 50 years ago. Particular attention has been paid to defining the biochemical and physiological mechanisms involved in safener activity, not only to assist safener development and optimization, but also as a means of understanding and manipulating the mechanisms which contribute to herbicide selectivity and resistance. This paper provides a critical review of this work and identifies future research requirements.

2 HISTORY OF HERBICIDE SAFENERS

The phenomenon of herbicide safening was discovered in 1947, following observations that tomato plants treated with 2,4,6-T did not suffer damage following accidental exposure to vapour of the herbicide 2,4-D.[1] Further investigations revealed that foliar treatment with 2,4-D protected wheat from barban injury.[2] However, this antagonism could not be exploited, because seed-treatment with 2,4-D proved to be damaging to the crop, while foliar sprays also reduced activity in target weed species. Despite this initial failure, Hoffman recognized the potential significance of these interactions and undertook a screening programme to identify compounds with safener activity. This produced the first commercial herbicide safener, 1,8-naphthalic anhydride (NA), which was patented by the Gulf Oil Co in 1971 for the protection of maize from injury by thiocarbamate herbicides.[3] However, the launch of NA was economically flawed because it expanded the market for the proprietary herbicides of a competitor, Stauffer Chemical Co, who subsequently patented their own safener, dichlormid.[4] In contrast to NA, which is applied as a seed-treatment, dichlormid, with its superior selectivity, was formulated with the herbicide, thus reducing application costs. In the face of such competition, NA failed to achieve its commercial potential and, despite being active in a number of species and against a diverse selection of herbicides, it was withdrawn from the market.

Nevertheless, the discovery of NA initiated intensive industrial research, albeit aimed at the discovery of safeners providing protection against proprietary products. Such research has targeted major crop–weed–herbicide combinations and used empirical screening methods to identify chemical leads.[5] This approach led to the discovery of the oxime ether safeners, cyometrinil, oxabetrinil and fluxofenim, by Ciba-Geigy (now Novartis),[6] and the 2,4-disubstituted thiazole carboxylates, such as flurazole, by Monsanto.[7] Over the last 15 years, these have been followed by an array of compounds representing diverse chemistries as illustrated in Table 1 which lists those compounds which are, or have been, available as commercial products. Early compounds were typically active in cereal crops, such as maize, sorghum and rice,

* Correspondence to: Joanna Davies, IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, UK
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Table 1. Herbicide safeners available as commercial products

Safener	Crop	Herbicide	Application method
Benoxacor (CGA 154281)	Maize	Metolachlor	Spray as mixture with herbicide
Cloquintocetmexyl (CGA 184927)	Wheat	Clodinafop-propargyl	Spray as mixture with herbicide
Cyometrinil (CGA 43089)	Sorghum	Metolachlor	Seed-treatment
Dichlormid (DDCA, R25788)	Maize	EPTC, butylate, vernolate	Pre-plant incorporated with herbicide
Fenchlorazole-ethyl (HOE 70542)	Wheat	Fenoxaprop-ethyl	Spray as mixture with herbicide
Fenclorim (CGA 123407)	Rice	Pretilachlor	Spray as mixture with herbicide
Flurazole (MON 4606)	Sorghum	Alachlor	Seed-treatment
Fluxofenim (CGA 133205)	Sorghum	Metolachlor	Seed-treatment
Furilazole (MON 13900)	Cereals	Halosulfuron-methyl	Spray as mixture with herbicide
Mefenpyr-diethyl	Wheat, rye, triticale, barley	Fenoxaprop-ethyl	Spray as mixture with herbicide
MG 191	Maize	Thiocarbamates	Spray as mixture with herbicide
Naphthalic anhydride (NA)	Maize	EPTC, butylate, vernolate	Seed-treatment
Oxabetrinil (CGA 92194)	Sorghum	Metolachlor	Seed-treatment

while protection was only provided against pre-emergence applications of herbicides representing a narrow spectrum of chemistries and modes of action, namely the thiocarbamate and chloroacetanilide classes. However, trends towards post-emergence herbicide treatments and the use of high-activity herbicide molecules, have led to the commercialization of safeners that protect winter cereals against post-emergence applications of aryloxyphenoxypropionate compounds, while safener activity is now widely reported against sulfonylurea, imidazolinone, cyclohexanedione and isoxazolidinone herbicides.[8] The majority of these compounds are marketed as mixtures with herbicides, although selective placement of the

safener as a seed-treatment may be necessary where greater crop–weed selectivity is required.

3 COMMERCIAL USES FOR HERBICIDE SAFENERS

With their ability to enhance herbicide selectivity between crop and weed, safeners have many potential uses. These include protection of crops from damaging levels of pesticide residues, thus allowing greater flexibility in the choice of crops grown in rotation, facilitating the use of higher herbicide rates to achieve more effective weed control, and the use of herbicides under adverse environmental conditions where crop damage is likely to occur. However, with environmental and economic pressures to minimize pesticide usage, the major role of herbicide safeners is seen as extending the use patterns of currently available herbicides and encouraging the development of molecules with favourable toxicological profiles, whose use would otherwise be limited by poor selectivity.

In addition, safeners have been used to address difficult weed control problems which, for technical and economic reasons, are unlikely to be solved by the development of conventional selective herbicides. For example, herbicide safeners may facilitate the control of weeds in botanically related crops. This possibility has been addressed by Codd[9] who investigated the ability of NA to protect cultivated oats (*Avena sativa* L) against diclofop-methyl injury with a view to controlling wild oat (*Avena fatua* L) infestations. Other crop–weed associations where safeners may provide a successful means of weed control include shattercane in grain sorghum (both *Sorghum bicolor* (L) Moench) and wild rice in cultivated rice (both *Oryza sativa* L).[5] Similarly, safeners may also prove a useful weed-control tool in crop-rotation systems where the previous crop appears as a 'volunteer' weed in following crops.[10] In particular, volunteer wheat and barley problems encountered in the barley–wheat rotations widely adopted in Europe may benefit from the use of herbicide safeners.[11] Safeners may also provide weed-control options for minor crops, which, due to their small market value, are not generally targeted for the development of new products. This possibility is illustrated by Mersie and Parker[12] who, on screening herbicides for the control of grass weeds in the cereal crop, teff (*Eragrostis tef* (Zucc) Trotter), found that selectivity could be achieved using NA seed-treatments in combination with pre-emergence chlorsulfuron applications.

The commercial viability of safener applications is clearly reflected in the number of herbicide-safener products now on the market (Table 1). However, possibly their most important application lies in their use as powerful research tools with which to identify and manipulate the biochemical and physiological mechanisms controlling herbicide selectivity. It is for this reason that considerable attention has been devoted to the study of safener mode of action.

Safeners act by reducing the ability of herbicides to reach and inhibit their target sites. This may be achieved through safener interactions with herbicide target sites or other receptor proteins involved in herbicide activity. Alternatively, safeners may reduce the amount of herbicide reaching its target in an active form, either by the direct chemical reaction of the safener with the herbicide molecule, by safener-induced reductions in herbicide uptake or translocation, or by safener-enhanced metabolism of herbicides to less active or immobile metabolites.

4 SAFENER INTERACTIONS WITH HERBICIDE RECEPTORS AND TARGET SITES

Several authors document the ability of safeners to counteract the effects of herbicides on those biochemical processes whose inhibition would normally lead to phytotoxicity. In particular, dichlormid has been reported to prevent EPTC-induced inhibition of fatty acid,[13] gibberellin,[14] acetyl coenzyme A,[15] carotenoid[16] and epicuticular wax biosynthesis.[17,18] Herbicide-induced inhibition of fatty acid and epicuticular wax synthesis is also counteracted by NA, MG 191 and AD 67,[13,17,18] and the oxime ether safeners.[19,20] These observations suggest that safeners may compete with herbicides for binding sites on critical herbicide receptor proteins, enhance the synthesis of target enzymes or reduce their susceptibility to herbicide inhibition through induction of less sensitive isozymes.

4.1 Competition of safeners with herbicides for binding sites on receptor proteins

The structural similarity of several herbicide-safener combinations has led to suggestions that safeners compete with herbicide molecules for binding sites on receptor or target proteins.[21] Taylor and Loader[22] proposed that antagonism of diclofop-methyl activity by 2,4-D in oats was a consequence of their physicochemical similarities, while similar proposals were made to account for the antagonism of EPTC activity by dichlormid.[23] However, where isolation of known herbicide receptors has been possible, safeners have not directly influenced herbicide interactions with these sites. For example, Polge *et al*[24] found that treatment of maize acetolactate synthase (ALS) extracts with NA did not prevent its inhibition by chlorsulfuron. Similarly, Köcher *et al*[25] and Hatzios[26] demonstrated that in-vitro inhibition of acetyl coenzyme A carboxylase (ACCase) by fenoxaprop-ethyl and sethoxydim cannot be reduced by the safeners fenchlorazole-ethyl and dichlormid, respectively.

In contrast, Walton and Casida[27] document the binding of a dichloroacetamide safener to a soluble maize protein (SafBP) and competitive inhibition of this binding by related dichloroacetamide safeners, the chloroacetanilide, metolachlor, and the thiocarbamate, EPTC. Furthermore, in-vitro inhibition of this binding activity was strongly correlated with safener effectiveness, while distribution studies showed that

SafBP was absent from species which do not respond to safener treatments.[28] The proposed identity of SafBP as an *O*-methyl transferase involved in lignin biosynthesis remains to be confirmed, but is consistent with the observation that chloroacetanilide and thiocarbamate herbicides inhibit lignification. The potential role of these observations in safener activity is discussed later.

4.2 Effects of safeners on the activity and sensitivity of herbicide targets

Several authors have investigated the effects of safeners on herbicide targets, particularly acetolactate synthase (ALS), which is the first enzyme of branched-chain amino acid biosynthesis and target for sulfonylurea and imidazolinone herbicides. For example, Rubin and Casida[29] observed a 25% increase in ALS activity of etiolated maize root and shoot tissue following treatment with dichlormid, while NA and oxabetrinil were found to elevate ALS levels in maize[30,31] and etiolated sorghum.[32] Furthermore, Milhomme *et al*[31] reported that ALS extracts prepared from the roots of safened seedlings were less sensitive to inhibition by imazaquin and metsulfuron-methyl. However, the significance of these observations is undermined by a series of contradictory reports. Further investigations by Barrett[33] revealed that neither NA, oxabetrinil, flurazole nor dichlormid enhanced ALS activity in chlorophyllous maize and sorghum seedlings. Furthermore, Polge *et al*[24] found that the two-fold increase in ALS activity of maize seedlings which accompanied NA and dichlormid treatments was offset by a doubling in the sensitivity of ALS to inhibition by chlorsulfuron. In contrast, the activity and sensitivity of maize ALS to sulfonylurea inhibition was not affected by NA[34] or BAS 145138 treatments.[35,36]

Because of the contradictory nature of these observations, the interaction of safeners with herbicide targets is not considered to be the primary mechanism of safener action. Certainly, Hatzios[37] concluded that such interactions cannot account for the specificity of many effective herbicide–safener–crop combinations. For example, safener competition for herbicide binding sites does not explain the ability of structurally unrelated safener molecules to provide protection against a specific herbicide or the ability of a single safener, such as NA, to prevent injury from herbicides with different modes of action. However, a biochemical stress, such as the interactions of both herbicides and safeners with SafBP or ALS,[27,38] may be the first stage of a signal transduction pathway leading to the transcriptional activation of genes involved in herbicide resistance. Hershey and Stoner[39] reported induction of a mRNA species of unknown function, designated *In2-2*, following treatment of maize with the benzenesulfonamide safener, *N*-(aminocarbonyl)-2-chlorobenzenesulfonamide (2-CBSU). De Veylder *et al*[38] also demonstrated that the *In2-2* gene could be activated in transgenic tobacco plants by treatment with chlorsulfuron or branched-chain amino acids, which are known to inhibit ALS activity *via* a negative feedback mechanism. Furthermore, induction of *In2-2* by chlorsulfuron was less pronounced in a tobacco line modified with a sulfonylurea-resistant form of ALS, while safener-induced growth retardation was also reduced in this line. These results not only imply that safeners interact with ALS, but also that transcriptional activation of the *In2-2* gene results from ALS inhibition. Definition of the pathway from recognition of ALS inhibition to gene activation is hindered by the unknown function of the *In2-2* protein. Nevertheless, this is the first study to propose a coherent role for the involvement of herbicide receptors in safener activity.

5 CHEMICAL ANTAGONISM

Theoretically, safeners may react with herbicide molecules to form an inactive or immobile complex incapable of causing herbicidal damage. This is the basis for the activity of herbicide adsorbents, such as activated charcoal and the lignin by-product PC 671, which form an external barrier between the crop and herbicide, thereby providing protection against soil-applied herbicides.[40] Chang[41] proposed that the oxime ether safeners may complex chloroacetanilide herbicides inside the crop plant. However, this does not account for the selective nature of tank-mixed safeners.

6 SAFENER EFFECTS ON HERBICIDE UPTAKE AND TRANSLOCATION

Following realization that several herbicides and their safeners have a common site of entry *via* the coleoptile of grass species, it was proposed that safeners may reduce herbicide uptake or translocation. Ezra *et al*[42] suggested that structural similarities between some herbicides and their safeners would lead to their competition for active sites of uptake. However, investigations of safener effects on these processes has produced a series of contradictory results (Table 2). The majority of workers have found that herbicide uptake is unaffected or enhanced by safener treatments. Where such changes have been observed, they are usually considered to be a consequence of safener interactions with other processes. For example, the cyometrinil-induced reduction in metolachlor uptake observed by Ketchersid *et al*[50] may be attributed to the decrease in transpiration rate which accompanies safener treatment. This itself reflects the ability of cyometrinil to prevent the inhibition of epicuticular wax formation associated with metolachlor treatment.[19] Similarly, reductions in the translocation of the imidazolinones imazethapyr and AC 263222 from maize roots to shoots following NA-treatment are believed to be a consequence of NA enhancement of herbicide metabolism to more polar and, therefore, less mobile metabolites.[65–67] Likewise, Fuerst and Lamoureux[68] concluded that reduced translocation of

Table 2. Effect of herbicide safeners on herbicide uptake

Safener	Herbicide	Species	Reference	Effect
AD 67	Acetochlor	Maize	43	↑
BAS 145138	Metazachlor	Maize	44	↓
			45	↑
Benoxacor	Metolachlor	Maize	46, 47	–
	Primisulfuron		48	–
Cloquintocet	Primisulfuron	Maize	48	–
Cyometrinil	Alachlor	Sorghum	49	–
	Metolachlor	Sorghum	49	–
			50	↓
			51	↑
	EPTC	Maize	42	↑
Dichlormid	Acetochlor	Maize	43, 45	↑
	Metazachlor			
	Metolachlor	Sorghum	51	↑
	EPTC	Maize	42	↓
			52	–
			53, 54	↑
Daimuron	Bensulfuron-methyl	Rice	55	↓
Fenchlorazole-ethyl	Fenoxaprop-ethyl	Wheat	25	–
Fenclorim	Metolachlor	Sorghum	56	–
	Pretilachlor	Rice	57	↓
Flurazole	Acetochlor	Sorghum	58	–
	Alachlor	Sorghum	59	↑
	Metolachlor	Sorghum	51	↑
	Metazachlor	Maize	45	↑
Fluxofenim	Metolachlor	Sorghum	60	–
Furilazole	Primisulfuron	Maize	48	–
MG 191	Acetochlor	Maize	43	↑
NA	EPTC	Maize	61	–
			42	↑
	Metolachlor	Sorghum	62	↓
			51	↑
	Barban	Oats	63	↓
	Metsulfuron-methyl	Maize	30	↑
	Primisulfuron	Maize	64	–
	Imazethapyr	Maize	65	–
Oxabetrinil	Metolachlor	Sorghum	20, 51, 60	↑
			56	–

Key: ↓ reduced uptake; – no effect; ↑ enhanced uptake.

metazachlor following BAS 145138 treatment was also due to increased metabolism. Additional evidence undermining the importance of safener effects on herbicide uptake is presented by several authors, who document the ability of safeners to elicit protective effects when applied after the herbicide.[69–71] Under these circumstances, opportunities for safener interference with herbicide uptake processes are eliminated.

7 EFFECTS OF HERBICIDE SAFENERS ON HERBICIDE METABOLISM

7.1 Plant metabolism of herbicides

Most plant species possess a three-phase detoxification mechanism, involving initial oxidative degradation of parent molecules during Phase I, followed by conjugation with endogenous substrates, such as glutathione during Phase II. In several cases, parent molecules may bypass Phase I and enter Phase II directly. Although such reactions usually serve to reduce the phytotoxicity and mobility of xenobiotics, Phase I oxidation is required for the activation of herbicides such as EPTC. During Phase III, Phase II metabolites may undergo further conjugation to produce insoluble residues, which are sequestered in vacuoles or bound in lignin biopolymers. Alternatively, Phase II conjugates can be directly sequestered in the vacuole by the activity of glutathione-conjugate pumps located in the vacuolar membrane.[72]

7.2 Phase I reactions

Although reduction and hydrolysis of herbicide molecules may occur during Phase I metabolism, the majority of reactions involve oxidations, such as alkyl oxidation, dealkylation, hydroxylation, epoxidation and sulfoxidation.[73] Many of these reactions are known to be catalyzed by cytochrome P_{450}-dependent mono-oxygenases. These are haemoproteins associated with the smooth endoplasmic reticulum and characterized by their ability to bind carbon monoxide, producing an absorption maximum at 450 nm.[74] In its oxidized state, the haem group can also bind substrates, XH, which are then oxidized, as illustrated in Fig 1.

As a result of intensive research over the past 40 years, bacterial and mammalian cytochrome P_{450} mono-oxygenase systems are known to be encoded by a highly divergent gene superfamily consisting of 65 gene families containing in excess of 450 sequences.[75] Microsomal and mitochondrial P_{450} systems have been isolated from a wide range of tissues, where they are responsible for the oxidative reactions of lipid, steroid, bile acid and vitamin D biosynthesis.[76] The mammalian liver P_{450} system has been demonstrated to catalyze the oxidation of over 1000 xenobiotic substrates, including many drugs and compounds of pharmacological interest.[77] Furthermore, many of these systems are known to be inducible by pretreatment with potential substrates and other xenobiotics. In contrast, plant cytochrome P_{450} systems have proved more difficult to characterize. Nevertheless, recent reviews cite evidence for the existence of over 50 systems in plants, including biosynthetic systems involved in the oxidative reactions of sterol, terpene, isoflavonoid, gibberellin, abscisic acid, cytokinin and lignin biosynthesis, and detoxification systems involved in the metabolism of xenobiotics such as herbicides.[78] Indeed, definitive in-vitro P_{450}-mediated metabolism has been demonstrated for the dealkylation of monuron,[79] chlorotoluron,[80] metolachlor[81] and alachlor,[82] as well as the hydroxylation of bentazone,[83] chlorotoluron,[80] 2,4-D,[84] diclofop,[85,86] flumetsulam,[87] primisulfuron,[88] prosulfuron[89] and triasulfuron.[90] Oxidative metabolism of these herbicides is performed by microsomal fractions, which

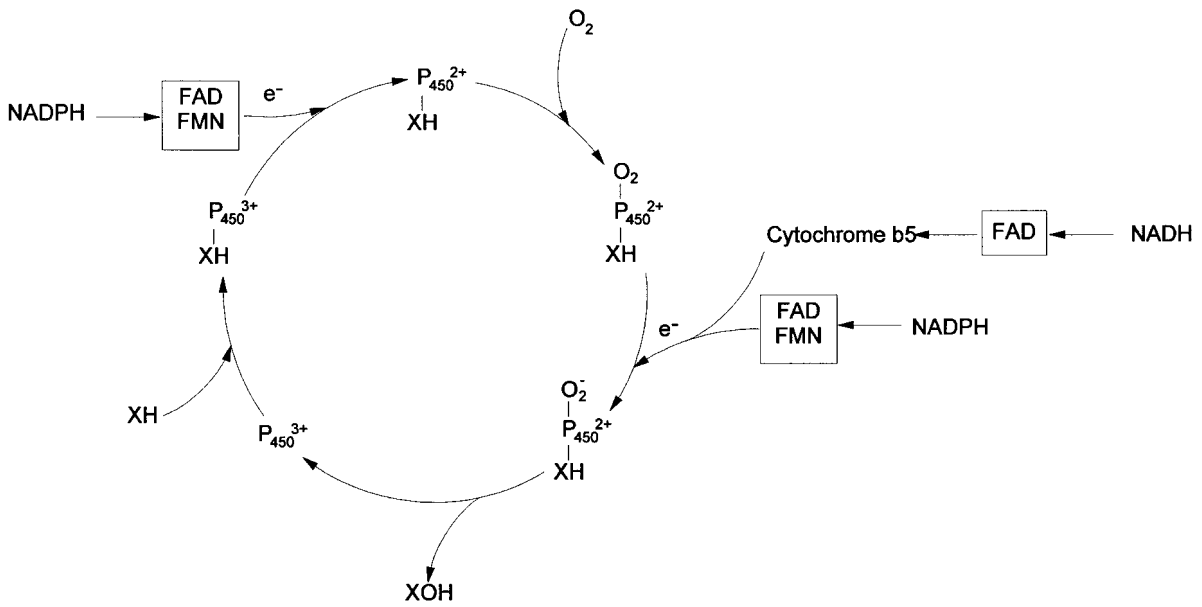

Figure 1. Reaction mechanism of cytochrome P_{450}. XH – substrate; XOH – oxidized substrate; FAD/FMN – NADPH cytochrome P_{450} reductase; FAD – NADH cytochrome b5 reductase.

require NADPH and molecular oxygen for activity whilst being susceptible to inhibition by carbon monoxide (CO) and inhibitors, such as 1-aminobenzotriazole (ABT), piperonyl butoxide and tetcyclacis. Conclusive evidence for the role of cytochrome P_{450} in herbicide metabolism is also provided by Topal *et al*[91] and Thalacker *et al*[92] who succeeded in purifying cytochrome P_{450}-dependent mono-oxygenases capable of 2,4-D and triasulfuron hydroxylation, respectively. Both studies demonstrated herbicide metabolism by the reconstituted enzyme systems. Reviews of this work are provided by Barrett[93] and Werck-Reichhart.[94]

Some Phase I reactions are believed to be catalyzed by peroxidases (EC 1.11.1.7). Unlike cytochrome P_{450}, these enzymes are widely distributed in plant tissues at high concentrations and catalyze oxidative reactions using hydrogen peroxide. As such, peroxidases are believed to be involved in proline hydroxylation, indole acetic acid oxidation, and lignification. They have also been implicated in the metabolism of aniline compounds produced on the degradation of phenylcarbamate, phenylurea and acyl aniline herbicides.[72]

7.3 Phase II reactions

During Phase II, herbicides or their Phase I metabolites undergo conjugation with endogenous substrates, such as glucose, amino acids or, more commonly, glutathione. Due to its role in the storage and transport of sulfur, reduced glutathione (GSH), is widely distributed in plant tissues, where it functions as a free-radical scavenger, thus protecting cells from oxidative damage.[95] The synthesis of this tripeptide from cysteine can be induced by the presence of active oxygen species, which cause its oxidation to glutathione disulfide (GSSG) (Fig 2). Reversion to GSH is catalyzed by glutathione reductase (EC 1.6.4.2) and requires NADPH. The conjugation of GSH with endogenous substrates or xenobiotics is catalyzed by glutathione *S*-transferases (GST; EC 2.5.1.18). These enzymes are cytosolic, homo- or heterodimers with

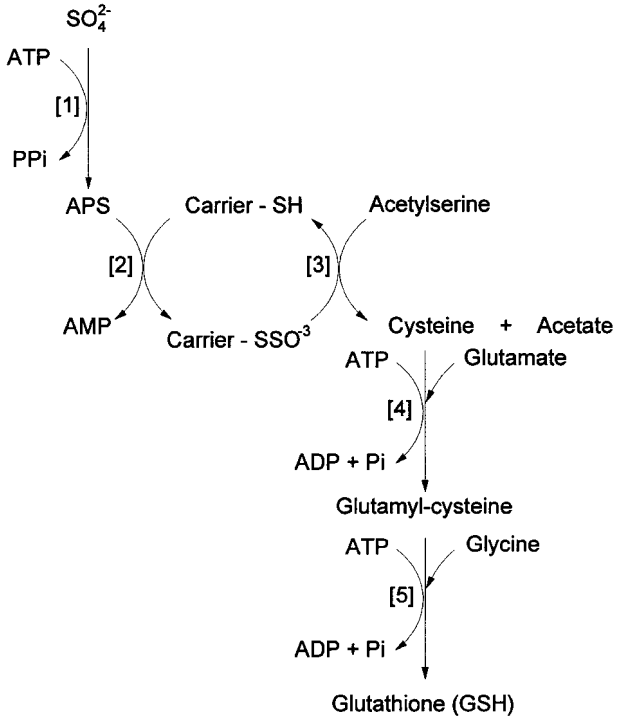

Figure 2. Pathway of glutathione synthesis in plants. 1. ATP sulfurylase; sulfate adenylyltransferase; EC 2.7.7.4. 2. APSSTase; adenosine 5-phosphosulfate sulfotransferase. 3. cysteine synthase; acetylserine sulfhydrylase; EC 4.2.99.8. 4. glutamyl-cysteine synthetase; GSH synthetase I; EC 6.3.2.2. 5. GSH synthetase II; EC 6.3.2.3. APS: adenosine 5-phosphosulfate (adenylylsulfate). ATP: adenosine triphoshate. AMP: adenosine monophosphate.

subunits in the range of 23–29 kDa and are often subject to regulation by auxins. To date, 35 genes encoding GST isozymes have been identified from 13 plant species, while seven isozymes are known to be involved in herbicide metabolism in maize.[95] These are composed of different combinations of five distinct subunits designated I, II, III, V and VI, and they have been named according to their subunit composition, ie GST I-I, GST I-II, GST I-III, GST II-II and GST II-III.[96]

Alternatively, some phenylcarbamate, phenylurea, sulfonylurea and imidazolinone herbicides undergo conjugation with glucose during Phase II. These reactions are usually catalyzed by glucosyl transferases (EC 2.4.1.71), using uridine diphosphate glucose (UDPG) as the glucosyl donor. In contrast, acidic herbicide molecules, such as the phenoxyacetic acids, may be subject to conjugation with the amino acids glutamine, valine, leucine, phenylalanine or trytophan. However, the enzymology of these reactions is largely uncharacterized.[73]

The relative ability of plant species to perform Phase I and II reactions is frequently correlated with their susceptibility to herbicide damage and, therefore, partly determines herbicide selectivity. Consequently, the ability of safeners to influence metabolic processes has been widely investigated as a possible mode of safener action.

Table 3. Studies demonstrating safener enhancement of oxidative herbicide metabolism *in vivo*

Safener	*Herbicide*	*Species*	*Reference*
BAS 145138	Chlorimuron-ethyl, primisulfuron, nicosulfuron	Maize	35
		Maize	36
Cyometrinil	Chlorsulfuron, metsulfuron-methyl, sulfometuron-methyl	Maize, wheat	97
Dimepiperate	Bensulfuron-methyl	Rice	101
Daimuron	Bensulfuron-methyl	Rice	55
Fenchlorazole-ethyl	Fenoxaprop-ethyl	Barley, wheat	102, 103
Flurazole	Alachlor, primisulfuron	Sorghum	104
		Maize	64
Fluxofenim	Bentazone	Sorghum	105
Oxabetrinil		species	
Furilazole	Primisulfuron	Maize	48
NA	Bentazone	Maize	83
		Sorghum	105, 106
	chlorsulfuron, metsulfuron-methyl, sulfometuron-methyl	Maize, wheat	97
	flumetsulam	Maize	87
	imazethapyr	Maize	70, 107
	AC 263222	Maize	67

7.4 Effects of herbicide safeners on Phase I metabolism

7.4.1 Cytochrome P_{450} mono-oxygenases

Herbicide safeners have been shown to enhance crop tolerance to several herbicides subject to the oxidative reactions of Phase I metabolism. In particular, NA, cyometrinil, dichlormid and BAS 145138 protect maize against sulfonylurea injury,[30,35,97] while NA also enhances the tolerance of maize to imidazolinone herbicides.[65,67] These observations suggest that safeners may act by enhancing the activity of oxidative enzyme systems such as cytochrome P_{450}. This theory was originally proposed by Leavitt and Pennel,[98] who suggested that dichlormid may protect maize from EPTC injury by stimulating the rate of EPTC sulfoxidation. This was echoed by Dutka and Komives,[99] who reported the synergistic effects of the mono-oxygenase inhibitors piperonyl butoxide and SKF 525A on EPTC activity in the presence of dichlormid. Similarly, Hatzios[100] demonstrated that metolachlor activity in grain sorghum could be synergized by the anti-oxidants ozone, piperonyl butoxide and propyl gallate in the presence of the safener, cyometrinil. These early speculative observations were confirmed when Sweetser[97] demonstrated the ability of NA and dichlormid to accelerate the oxidative metabolism of chlorsulfuron, metsulfuron-methyl and sulfometuron-methyl in maize. Further evidence has since been provided by numerous workers who, following observation of safener-enhanced oxidative metabolism *in vivo* and inhibition of metabolism by known cytochrome P_{450} inhibitors, have concluded that safeners induce cytochrome P_{450} mono-oxygenases responsible for herbicide degradation. A summary of these reports is provided in Table 3.

In many cases, these observations have been followed by reports of safener-enhanced herbicide metabolism by microsomal enzyme systems with the characteristics of cytochrome P_{450} mono-oxygenases, (Table 4). Most striking of these are reports by Fonne-Pfister and Kreuz,[109] who observed a 15-fold increase in the chlorotoluron hydroxylase activity of maize microsomes following seed-treatment with benoxacor. Similarly, McFadden *et al*[83] found that seed-treatment with NA was essential for the detection of bentazone hydroxylase activity in microsomes extracted from etiolated maize shoots, while Frear and Swanson[89] observed a 28-fold increase in prosulfuron hydroxylation by wheat microsomes following treatment with NA or cloquintocet-mexyl.

Additional evidence implicating cytochrome P_{450} in safener action has been sought by assessing the effects of safener treatments on total cytochrome P_{450} contents and levels of microsomal electron transfer components. However, such investigations have produced variable results. For example, while the cytochrome P_{450} contents of maize seedlings were significantly enhanced by pretreatment with NA[86,107] and benoxacor,[88,109] studies conducted by McFadden *et al*,[83] Frear *et al*[87,90] and Moreland and Corbin[105] indicated that NA had no significant effect on total

Table 4. Studies demonstrating safener enhancement of NADPH-dependent microsomal herbicide metabolism

Safener	*Herbicide*	*Species*	*Reference*
BAS 145138	Bentazone	Sorghum	108
Benoxacor	Bentazone	Sorghum	108
	Chlorotoluron	Maize	109
	Primisulfuron		88
Cloquintocet-mexyl	Prosulfuron	Wheat	89
Cyometrinil	Chlorotoluron	Wheat	80
Dichlormid	Bentazone	Sorghum	108
Flurazole	Bentazone	Sorghum	108
Fluxofenim	Bentazone	Sorghum	108
	Chlorotoluron	Wheat	80
MG 191	Acetochlor	Maize	110
NA	Acetochlor	Maize	110
	Metolachlor	Maize	111
	Bentazone	Maize	83, 111
		Sorghum	105, 106, 108
	Chlorimuron-ethyl	Maize	112
	Diclofop-methyl	Wheat	86
	Chlorotoluron	Wheat	86, 90
	Chlorsulfuron	Wheat	90
	Prosulfuron	Sorghum	113
		Wheat	89
		Maize	113
	Primisulfuron	Maize	111
	Triasulfuron	Maize	111, 114
		Wheat	90, 92
	Flumetsulam	Maize	87
	Imazethapyr	Maize	115
	AC 263222	Maize	67
Oxabetrinil	Bentazone	Sorghum	105, 108
	Chlorotoluron	Wheat	80

cytochrome P_{450} content or NADPH-cytochrome c reductase activity.

This lack of correlation between the effects of safeners on total cytochrome P_{450} content and rates of oxidative metabolism *in vitro* indicates that safeners may induce P_{450} isozymes specific for herbicide degradation at the expense of isozymes involved in the metabolism of endogenous substrates. Consequently, the effects of safeners on other cytochrome P_{450}-dependent activities have been measured to facilitate differentiation between isozymes of the cytochrome P_{450} pool. For example, Moreland *et al*[111] reported that NA pretreatments induced a 15-fold increase in the ability of maize microsomes to hydroxylate bentazone, whilst having little effect on the capacity for the hydroxylation of the endogenous substrates cinnamic and lauric acids. Furthermore, this study also demonstrated differential enhancement of activities for specific herbicide substrates, even within the same herbicide family. For example, hydroxylation of the sulfonylureas, nicosulfuron and triasulfuron, was enhanced by four- and ten-fold, respectively, following NA treatment. Similar observations have been reported by Zimmerlin and Durst,[86] Frear *et al*[90] and Moreland *et al*,[108] and suggest that safeners may induce isozymes for specific herbicide substrates.

Further definition of those isozymes involved in safener action is hindered by the complexity of P_{450}-based herbicide metabolism, as revealed by recent attempts to characterize the substrate specificities of P_{450} systems, using a combination of kinetic and competitive inhibition studies. For example, Frear *et al*[87] proposed that the hydroxylation of flumetsulam at two positions is catalyzed by two isozymes with distinct kinetic properties and inhibition responses but similar safener induction patterns. Conversely, competitive inhibition studies conducted by Barrett[93] suggest that the herbicides nicosulfuron, bentazone, chlorimuron-ethyl, imazethapyr and chlorotoluron may be metabolized by P_{450}s with common features or a common P_{450} with several binding sites. Furthermore, on investigating the inheritance of metabolic capabilities in maize, Barrett[93] found that nicosulfuron metabolism is controlled by a single gene, presumed to encode a P_{450}, which also controls bentazone and imazethapyr metabolism and is inducible by NA. However, hydroxylation of bentazone is also controlled by a second, distinct, non-inducible P_{450}. Thus, a single P_{450} may be capable of metabolizing several substrates, while multiple isozymes may be involved in the metabolism of a single substrate. These findings clearly indicate the powerful role that safeners play in characterizing oxidative systems involved in herbicide metabolism.

7.4.2 Peroxidases

Following observations that EPTC sulfoxide is more phytotoxic than EPTC itself, Blee[116] proposed that dichlormid could prevent herbicide injury by inhibiting the metabolism of EPTC to its sulfoxide. Further investigations revealed that pre-treatment with dichlormid inhibited EPTC sulfoxidation by a maize microsomal peroxidase. These observations agree with those of Harvey *et al*,[117] who reported that, on inhibiting maize peroxidase activity, dichlormid offset the stimulatory effects of EPTC on this enzyme. However, as few researchers have considered these possibilities, little evidence exists to support such claims.

7.5 Effect of herbicide safeners on Phase II metabolism

7.5.1 Glutathione conjugation

Herbicide safeners have been demonstrated to enhance crop tolerance to several chloroacetanilide and thiocarbamate herbicides known to undergo glutathione conjugation during Phase II. Investigations of safener effects on the metabolism of these herbicides revealed that the dichloroacetamide safeners dichlormid, benoxacor and BAS 145138 increase GSH conjugation of metolachlor, metazachlor and acetochlor,[43,46,47,51,68] while flurazole, NA and the oxime ethers have been shown to enhance conjugation of metolachlor.[51,60] Similarly, the conjugation of pretilachlor and acetochlor was increased by fenclorim and

AD 67, respectively,[118,143] while MG 191 enhanced the conjugation of acetochlor[43] and EPTC.[119] Furthermore, Tal *et al*[120] observed increases in the conjugation of the aryloxyphenoxypropionoate herbicide, fenoxaprop-ethyl, with GSH in wheat and barley seedlings treated with fenchlorazole-ethyl.

Many reports have been accompanied by observations of safener-induced promotion of GSH content. For example, dichlormid has been reported to increase GSH levels in maize,[43,53,121–125] sorghum[126] and tobacco.[127] Other safeners found to promote GSH content include BAS 145138,[43] benoxacor,[43,125,128] flurazole,[126,129] fenclorim,[118] MG 191[43,130] and AD 67.[43] In some cases, increases have been attributed to enhancement of glutathione reductase or enzymes involved in glutathione biosynthesis.[131] However, following the discovery that flurazole forms a GSH conjugate in maize, Breaux *et al*[134] proposed that such safener conjugates may enhance GSH production by binding to glutamyl-cysteine synthetase, thereby overriding its feedback inhibition by GSH. Such a mechanism has been observed in human red blood cells, where feedback inhibition was released on exposure to the GSH conjugate, *S*-2,4-dinitrophenyl glutathione.[135]

Although the ability of safeners to promote GSH synthesis is well established, its direct relevance to safener action remains questionable for two reasons. Firstly, enhancement of GSH content does not always coincide with herbicide conjugation. For example, Ezra and Gressel[121] found that, although dichlormid-induced increases in GSH content of maize cells were detectable 12 hours after treatment, EPTC conjugation was almost complete within eight hours. Secondly, safener efficacy is not well correlated with elevated GSH levels. For example, despite its ability to protect maize and sorghum against EPTC injury, NA does not significantly affect the GSH content of these species.[124,126] Similarly, Lay and Niland[136] found that enhanced GSH levels were not always apparent in maize lines protected by dichlormid, while, conversely, increases were detected in maize lines not protected by dichlormid. Furthermore, although BAS 145138 enhanced glutathione conjugation of metazachlor in maize, Fuerst and Lamoureux[68] found no change in GSH content. However, as GSH can lead to an induction of GST activities,[95] promotion of GSH synthesis by safeners may be part of a signal transduction pathway leading to accelerated glutathione conjugation. Strong correlations exist between the efficacy of safener treatments and their ability to induce GST activities. A summary of studies demonstrating these effects is provided in Tables 5 and 6.

7.5.2 *Glucose conjugation*

Several imidazolinone and sulfonylurea herbicides whose activities are antagonized by safener treatments undergo conjugation with glucose. The ability of safeners to accelerate glucosylation was recognized by Kreuz *et al*,[154] who reported that cloquintocet-mexyl enhanced glucosylation of the aryloxyphenoxypropionate herbicide, clodinafop, in wheat. Similarly, BAS 145138 was found to increase the glucosylation of hydroxylated chlorimuron-ethyl in maize.[35] These authors proposed that such an effect may result from promotion of constitutive and/or inducible UDP-glucosyl transferase (EC 2.4.1.71) activities or the increased availability of glucose, due to enhanced UDP-glucose synthesis or modulation of β-glucosidase activity. However, the effects of safeners on these processes are yet to be defined.

Table 5. Studies demonstrating safener promotion of enzymes involved in glutathione synthesis

Enzyme	*Safener*	*Species*	*Reference*
ATP sulfurylase (EC 2.7.7.4)	Dichlormid	Maize	122
	Benoxacor	Maize	132
APSSTase	Dichlormid	Maize	132
	Benoxacor	Maize	132
GSH synthetase (EC 6.3.2.3)	Dichlormid	Maize	53
		Tobacco	127
Glutathione reductase (EC 1.6.4.2)	Dichlormid	Maize	123
	MG 191	Maize	130
	Fluxofenim	Sorghum	133
	Fenclorim	Rice	118

8 MOLECULAR MECHANISMS OF SAFENER ACTION

Although enhancement of herbicide metabolism is now recognized as the predominant mechanism of safener activity, the molecular mechanisms and signal transduction pathway leading from recognition of safener presence to enhanced enzyme expression remain unknown.

Direct activation of metabolic enzymes by safener

Table 6. Studies demonstrating safener enhancement of GST activities

Safener	*Species*	*Reference*
AD 67	Maize	43
BAS 145138	Maize	43, 45, 68
Benoxacor	Maize	47, 125, 128, 137–141
Cloquintocet-mexyl	Wheat	142,143
Dichlormid	Maize	43,123–125, 136, 144–149
	Pea	150
	Sorghum	126,144
Fenchlorazole-ethyl	Wheat	151
Fenclorim	Rice	152
Flurazole	Maize	144,153
	Sorghum	126
Fluxofenim	Wheat	142
MG 191	Maize	43,130
NA	Maize	144
	Sorghum	126,144
Oxabetrinil	Sorghum	126

molecules has been eliminated as a possible mechanism, following the observation that dichlormid and oxabetrinil failed to enhance enzymatic conjugation when applied *in vitro*.[124,126] Instead, safeners are assumed to act at a transcriptional level by regulating gene expression. Evidence for this mode of action is provided by Wiegand *et al*,[153] who reported that flurazole induced a three-fold increase in the steady-state level of GST I/I mRNA, and Miller *et al*,[141] who observed reductions in the ability of benoxacor to induce the activity of a GST capable of metolachlor conjugation following treatment of maize cells with the protein synthesis inhibitor cycloheximide, and the mRNA synthesis inhibitor, cordycepin.

Hatzios[37] proposed a model which assumes the existence of activator or repressor proteins that interact with regulatory elements within the promoter region of metabolic genes. When present, safener molecules would modulate the activity of these transcription factors. Evidence for the interaction of safeners with such regulatory mechanisms has not been forthcoming but future work may benefit from consideration of the nature of other stimuli which also lead to induction of metabolic activities.

Some plant GST genes are known to possess *ocs* (octopine synthase) elements within their promoter regions. Possession of this element confers GST inducibility by a variety of electrophilic agents, including plant hormones, heavy metals, pathogen attack, wounding and environmental conditions which generate oxidative stress.[155] Certainly, the soybean GST encoded by the GH2/4 gene has a promoter that is activated by a wide range of chemical agents, including auxins, salicylic acid, abscisic acid, jasmonic acid, heavy metals, hydrogen peroxide, glutathione and also heat shock.[156] The diverse nature of these chemical and environmental stressors suggests that each evokes a common response which subsequently leads to GST induction. As several of these agents are known to alter levels of reactive oxygen species or cause oxidative damage directly, it seems likely that the transcription of plant GSTs is controlled by changes in redox status. Under these circumstances, induction of GST activities is believed to be a defence mechanism, protecting cells from membrane lipid peroxidation and oxidative DNA damage. Such a mechanism is analogous to that seen in animal GSTs, where promoter regions contain antioxidant-responsive or electrophile-responsive elements, which provide binding sites for the activator protein-1 transcription complex (AP-1). Binding of this transcription factor is essential for gene expression but is subject to regulation by changes in redox state.

This mechanism suggests that auxins are able to influence the production of reactive oxygen possibly by stimulation of NADPH or NADH oxidase, or effects on cell division and elongation.[95] Whether safener induction of GST activity also results from changes in reactive oxygen levels remains to be questioned. Interestingly, there are great similarities between the effects of auxins and safeners. For example, synthetic and natural auxins are reported to have safening properties[34] while significant sequence homology has been observed between safener-induced and auxin-regulated GSTs and proteins.[38,96,143] Comparisons between the promoter regions within these sequences will help establish whether auxins and safeners regulate GST expression *via* similar mechanisms.

9 HERBICIDE SAFENERS –THE FUTURE

While herbicide safeners will undoubtedly continue to provide new solutions to many weed control problems, perhaps their most powerful application lies in their use as tools with which to identify the metabolic pathways controlling herbicide selectivity. Characterization of the metabolic dichotomies between target and non-target plant species is essential if these are to be exploited in the development of new, environmentally safe and selective weed control strategies. This requires definition of substrate specificities of metabolic enzymes and isolation of the corresponding genes. Until recently, this has proved difficult, given the low titre, extreme lability and anticipated plethora of enzymes, such as the cytochrome P_{450} mono-oxygenases in plant tissue. However, Barrett[157] proposes that direct gene isolation may become routine in the near future. In contrast, identifying roles for gene products and assessing their relative contributions to herbicide selectivity will be difficult. Nevertheless, this may be partially resolved through the effects of safeners and, once such information is available, it will not only become possible to adopt a more rational approach to the development of environmentally safe weed control strategies but will also provide biochemical and molecular tools with wide-ranging applications.

In particular, isolation of genes encoding safener-inducible enzymes may facilitate the development of genetically modified crops with enhanced metabolic capacity. This principle has already been demonstrated in tobacco, where transformation with the rat P_{450}, CYP1A1 conferred resistance to chlorotoluron.[158,159] Furthermore, transgenic tobacco expressing CYP105A1 from *Streptomyces griseolus* (Waksman and Henrici), was capable of activating the sulfonylurea pro-herbicide, R7402, by *N*-dealkylation and showed greater capacity for chlorimuron-ethyl hydroxylation.[160] Efficient expression of these activities required fusion with yeast NADPH cytochrome P_{450} oxidoreductase in the case of CYP1A1, and targeting to the chloroplast for CYP105A1. In contrast, Werck-Reichhart[94] predicts that the expression of plant P_{450} genes will be more efficient. However, the work of Gaillard *et al*,[161] who demonstrated that treatment of barley with cloquintocet-mexyl not only accelerates GST activity but also enhances the activity of a vacuolar transporter for glutathione-metolachlor and glucoside-hydroxy-primisulfuron conjugates, suggests that co-expression with other factors may still be necessary to achieve efficient tolerance.

Whilst the development of genetically modified crops has commanded considerable commercial interest in recent years, the continued development of new herbicides and safeners will also benefit from characterization of those genes coding for herbicide-metabolizing enzymes. In particular, this will aid identification of herbicides likely to serve as substrates for detoxifying enzymes in crop plants and also safener molecules capable of inducing relevant metabolic activities. The selectivity of potential herbicides could be diagnosed from their susceptibility to enzymatic degradation *in vitro*, while bioassays could be developed whereby potential safeners are tested for their ability to induce critical activities or genes in crop plants.

Such an approach could be adopted to address specific needs, such as the need for safeners to protect dicotyledonous crops, given the diversification of arable systems away from cereal monocultures. Safener activity has been observed in broad-leaf crops, including potatoes and soybeans which can be protected from metribuzin injury by the growth retardants daminozide, triapenthenol and BAS 140810.[162] Similarly, the tolerance of oilseed rape to picloram can be enhanced by pre-treatment with the closely-related pyridine herbicide, clopyralid.[163] While neither of these observations has led to the development of a commercial product, the prediction of other beneficial interactions would benefit from characterization of metabolic pathways in dicotyledonous species. Certainly, there is some evidence to suggest that inducible metabolic activities responsible for safener activity in monocotyledons are also present in dicotyledonous crops. For example, the induction, in *Arabidopsis thaliana* (Heynh) plants transformed with only one chimeric gene, of the maize *In2-2* promoter by substituted benzensulfonamide safeners suggests that the induction pathway leading from safener application to *In2-2* expression is conserved between monocotyledonous and dicotyledonous species.[38] Furthermore, NA-inducible cytochrome P_{450} activities capable of degrading metolachlor, alachlor and bentazone, exist in mung bean (*Vigna radiata* L).[82] Similarly, work by Edwards[150] demonstrated the presence of a dichlormid-inducible GST capable of atrazine metabolism in pea (*Pisum sativum* L), albeit restricted to root tissue. This suggests that dichlormid may not be readily translocated to the shoot system, although this was not investigated further.

In fact, with the exception of Riden and Asbell,[164] Jablonkai and Dutka[165] and Yenne *et al*[60] who investigated the fate of NA, MG 191 and the oxime ether safeners oxabetrinil and fluxofenim, respectively, few publications have contemplated the issues of safener uptake, translocation and metabolism. These investigations are becoming essential now that safeners are subject to the same environmental risk assessment procedures as herbicides themselves. Furthermore, such research would lead to a greater understanding of those processes critical to safener activity, particularly as there is evidence to suggest that the ability of some safeners to enhance herbicide degradation may result from induction of their own metabolism *via* the same mechanisms.[60] Certainly, a mRNA species designated *In2-1*, isolated from maize, is known to be inducible by the benzenesulfonamide safener 2-CBSU and chlorsulfuron.[39] Thus investigation of safener metabolism, followed by comparisons with herbicide detoxification pathways, may provide an alternative approach to the prediction of beneficial herbicide–safener interactions.

Whilst being fundamental to herbicide and safener development, the process of isolating metabolic enzymes and genes will also generate probes, or biomarkers, which could be used as tools in their own right. For example, the availability of antibodies for specific enzymes known to be responsible for herbicide resistance, could lead to the development of field testing methods for the early detection of herbicide resistance problems in weeds. Riechers *et al*[142] have already demonstrated the potential use of enhanced GST, as measured by ELISA, as an indicator of dimethenamid tolerance, albeit in wheat. Furthermore, characterization of metabolic activities is hindered by detection difficulties, notably for the cytochrome P_{450}s, in older plant tissue. Certainly, the activity of safeners in older plants would suggest that P_{450}s are present in this material, although Hendry[166] and Davies[167] report a rapid decline in microsomal activities shortly after germination. This anomaly reflects the need to use young, achlorophyllous seedlings for the measurement of P_{450} by spectrophotometric methods. The use of probes would enable detection of responses in older, chlorophyllous material and aid prediction of herbicide selectivity and safener activity in relevant plant material. Such an approach is already used during environmental risk assessment procedures where there is a need for methods of detecting pollutants, particularly pesticides, in complex water or effluent samples. The ability of a sample to induce a metabolic response, specifically enhanced P_{450} activity, has been used as an indication of xenobiotic stress in fish and is currently being assessed in plants.[168]

Finally, inducible gene expression systems, such as those regulating the production of proteins in transgenic organisms, have long been recognized as a tool for understanding basic biological processes. Inducible systems are based on the transformation of an organism with a structural gene of interest under the transcriptional control of a promoter known to be responsive to a specific inducing stimulus.[169] Hershey and Stoner[39] recognized the potential for development of a novel regulatable gene expression system based on a safener working in combination with a promoter derived from a safener-responsive gene. For this purpose, they undertook isolation and characterization of cDNA clones for RNA species, designated *In2-1* and *In2-2*, induced by the safener, 2-CBSU in maize. A chimeric gene incorporating the *In2-2* promoter and the *Bacillus amyloliquefaciens SacB* gene, which directs

fructan synthesis to the cytosol, was then introduced into tobacco. Subsequent treatment of transgenic plants with 2-CBSU enabled the regulation of fructan synthesis in the cytosol and, thus, the study of the effect of fructan accumulation on plant development.[170]

10 CONCLUSIONS

It is now widely accepted that herbicide safeners enhance crop tolerance by regulating the expression of genes involved in herbicide metabolism. With this mode of action, safeners provide a relatively inexpensive and flexible method of improving herbicide selectivity, without incurring the ecological risks perceived to be associated with resistant varieties. As such, they will undoubtedly continue to provide new solutions to many weed control problems, while their ability to modulate the metabolic pathways controlling herbicide selectivity makes them powerful research tools with wide-ranging applications. In particular, the continued development of environmentally safe crop protection products is dependent on fundamental knowledge of the dichotomies in herbicide metabolism between target and non-target plants. Herbicide safeners, with their unique mode of action, are vital tools in the aquisition of such knowledge.

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